Simultaneous Measurement of Several Cytokines Using Small Volumes of Biospecimens[1]

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Abstract

The role of host immunity in the development of virus-induced cancers has been difficult to elucidate, in part because of our inability to effectively measure multiple immune parameters using available amounts of biological material. The objective of the present study was to validate the use of a newly developed multiplex assay, the LINCOplex assay, for the simultaneous measurement of multiple cytokines [interleukin/(IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and tumor necrosis factor-α]. Supernatants obtained from peripheral blood mononuclear cell cultures stimulated with various different mitogens and antigens (including phytohemagglutinin, influenza, tetanus, HPV16 E6 and E7 peptides, and media alone) were selected for study. In total, 750 specimens obtained from 26 participants were tested in replicate using the 8-plex LINCOplex assay (25 μl of specimen required per well). Every specimen was included in duplicate in a blinded fashion. For some specimens, multiple 2-fold dilutions of the same specimen were included to evaluate the linearity of results. The availability of independently obtained IL-2 and IFN-γ results from standard single cytokine (simplex) assays allowed for a direct comparison between the LINCOplex results and those obtained from the simplex assays. Spearman correlation coefficients for continuous results, and exact agreement rates and weighted kappa statistics for quartiled variables, were computed to evaluate intra- and interassay agreement. IL-4 levels were consistently below the detectable level of the assay (3 pg/ml) whereas IL-6 and IL-8 levels were consistently above the highest detectable level of the assay (10,000 pg/ml), and these three cytokines were, therefore, not evaluated further. For the remaining five cytokines, excellent intra-assay reproducibility was observed, with Spearman correlation coefficients consistently above 0.90 for all five cytokines. Exact agreement rates ranging from 77.6–90.3% and weighted kappas ranging from 0.81–0.92 were observed. Similar results were observed when analysis was restricted to supernatants obtained from cultures that had been stimulated with HPV16 peptides and when analysis was restricted to supernatants obtained from cultures containing no antigen or mitogen, suggesting that the LINCOplex assay is applicable under conditions where moderate or weak cytokine responses/levels are expected. Linearity of results was observed when dilutions of a single specimen were blindly tested, with the exception of IL-2 and IL-10, where deviations from linearity were sometimes observed. For IL-2 and IFN-γ, where results from simplex assays were available for comparison, the LINCOplex assay and the simplex assay results agreed well. Spearman correlation coefficients were 0.86 and 0.93 for IL-2 and IFN-γ, respectively. Exact agreement and weighted kappa values were 68.5% and 0.72 (95% confidence interval, 0.65–0.79), respectively, for IL-2 and 67.3% and 0.73 (95% confidence interval, 0.67–0.80), respectively, for IFN-γ. These results indicate the applicability of the LINCOplex assay for the simultaneous measurement of multiple cytokines using small amounts of biological material.

Introduction

HPVs,[3] EBV, hepatitis B virus, and hepatitis C virus are all common viral infections that uncommonly result in the development of neoplasia (1, 2). Studies aimed at understanding the factors that lead a small fraction of infected individuals to progress to more severe disease have increasingly focused on evaluating the immune response to these viruses (3–6). It is suggested that cancers caused by viruses arise as a result of inadequate control of the viral infection by the immune system. However, the exact nature of what distinguishes a successful *versus* an inadequate immune response is poorly understood. This is partly due to the complexity of the immune system and the difficulty in systematically evaluating immune parameters that may be important predictors of disease.

One important stumbling block is the availability of lim-

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[3] The abbreviations used are: HPV, human papillomavirus; PBMC, peripheral blood mononuclear cell; IL, interleukin; TNF, tumor necrosis factor; AIS, ALTS Immunology Study; PHA, phytohemagglutinin; CI, confidence interval; LSIL, low-grade squamous intraepithelial lesions; ALTS, ASCUS, LSIL Triage Study; ASCUS, atypical squamous cells of undetermined significance.

ited amounts of biological material collected in population-based epidemiological studies from which the immune response can be assessed. This same difficulty applies to the monitoring of volunteers in vaccination trials, where the elucidation of immunological markers of a successful (protective) immune response is of interest.

We and others have been interested in evaluating levels of various cytokines systemically and locally at the cervix, with the goal of elucidating parameters associated with a permissive or protective immune response against HPV infections and cervical neoplasia (4). To date, the relatively small amounts of biological material that can feasibly be collected from study participants has limited the number of immune parameters that can be assessed. For example, in studies that have examined *in vitro* responses to HPV antigens by PBMCs and correlated these responses with a degree of cervical neoplasia, we and others have commonly focused on the measurements of IFN-γ, IL-2, IL-5, and IL-10 as common markers of cell-mediated (IFN-γ and IL-2) and humoral (IL-5 and IL-10) immune responses (4). At the cervix, we have been interested in determining whether natural levels of cytokines in cervical secretions are predictive of disease risk among HPV-infected individuals (7, 8).[4] The volume of material collected from the cervix, however (on the order of 20–100 μl), has made a systematic evaluation of cytokine levels at the cervix impossible.

Recently, new techniques have been developed to allow for the measurement of multiple cytokines in a single assay using limited amounts of material (9, 10). One such assay, the LINCOplex test, was initially developed to measure up to eight different cytokines simultaneously using as little as 25 μl of material. This assay relies on the use of polystyrene beads, each with a unique signature mix of fluorescent dyes that can be discriminated by a laser-based detection instrument, the Luminex^{100} (Luminex Corp., Austin, TX). Each bead type is coated with a specific antibody to the cytokine of interest. At the time of our study, the LINCOplex assay had been developed for simultaneous testing for the following eight cytokines: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and TNF-α. It is theoretically possible that this same technology could eventually be used to measure several dozen cytokines in one assay.

Here, we describe results of a study designed to determine the reproducibility and validity of this new LINCOplex assay. PBMC culture supernatants obtained during the course of testing specimens from a large-scale clinical trial of nearly 5000 women with evidence of equivocal or low-grade cervical neoplasia (the ASCUS, LSIL, Triage Study of ALTS) were used. In total, 750 specimens were tested in replicate as part of this investigation. Within and between batch reproducibility, reproducibility at high and low levels of cytokines, and comparability with currently available single cytokine (simplex) assays were determined. Results suggest that the multiplex assay is highly reproducible and that it correlates very well with standard simplex assays.

Materials and Methods

The AIS Study. The AIS study is a longitudinal study designed to evaluate the role of host immune responses to HPV in the presence, persistence, and progression of HPV infection and its associated lesions. The study, part of a larger effort to evaluate various triage strategies for the management of equivocal and low-grade cervical neoplasia, consists of 5066 women recruited at four clinical sites throughout the United States. The design and methods of this larger trial have previously been published (11). To be eligible, women had to be referred to one of the study clinics with a diagnosis of equivocal or low-grade cervical lesion and agree to be randomized into one of three triage strategies being evaluated for the management of these equivocal and low-grade lesions. On a subset of 873 (63% of those eligible) women randomized into the observational arm of the trial (*i.e.,* no biopsy performed unless there was cytological evidence of progression), 50 ml of blood were collected and shipped fresh to our laboratory to permit real-time, functional monitoring of immune responses to HPV. Tests performed that are relevant to the present validation study include the measurement of IL-2 and IFN-γ production in response to stimulation with HPV16 E6 and E7 peptides and other antigens/mitogens. As described more fully below, for the present study, specimens obtained from a total of 26 individuals were selected.

Culture for IL-2 Bioassay. Peripheral blood was collected in Vacutainer tubes (Becton Dickinson), and PBMCs were isolated by gradient centrifugation using Ficoll-Hypaque. Cells were washed and resuspended in R2E tissue culture media (Biofluids, Rockville, MD) supplemented with 5% heat-inactivated pooled human AB serum (Sigma). Cultures were established in 96-well round bottom plates (Costar) with 300,000 cells plated into replicate wells in a final volume of 200 μl. Cells were cultured with media alone or in the presence of PHA-M (1:100; Life Technologies, Inc.), influenza virus (1:100; American Type Culture Collection), tetanus toxoid (20 μg/ml; Pasteur-Merieux), or pooled peptides of HPV-16 E6 (amino acids 1–158) or E7 (amino acids 1–98) as shown in Table 1. In general, the pools consisted of 15-mer peptides with 10 amino acid overlap at a stock concentration of 50 μM for each peptide, and in the assay the final concentration was 5 μM for each peptide. After 1 h at 37°C, anti-Tac was added to each well to prevent use of IL-2. A seed culture of the Hd245332 anti-Tac-producing cell line was generously provided by Dr. Thomas Waldmann (Metabolism Branch, NCI, Bethesda, MD). Cells were incubated for 7 days at 37°C. After 7 days, the culture supernatants were collected, transferred to a second 96-well plate, and frozen at −20°C.

CTLL-2 Bioassay for IL-2. IL-2 was detected in the thawed fluids from the cultures described above by performing a bioassay on the IL-2-dependent cell line CTLL-2. Supernatant fluids were diluted 1:4 or 1:8 and were added to CTLL-2 cells plated at a cell density of 8×10^4 cells/well. After 24 h, 1 μCi of ^{3}H-thymidine was added/well and cells were cultured for an additional 16–20 h. Cells were harvested, and ^{3}H-thymidine uptake was quantified on a liquid scintillation counter. Results were expressed in cpm.

Culture for Cytokine Induction. PBMCs (at a final concentration of 1.5×10^6/ml) were incubated in the presence of PHA-M (1:100), influenza virus (1:100; American Type Culture Collection), or the HPV E6 and E7 peptide pools (5 μM) for 7 days at 37°C and 6% CO2 in R2E media containing 5% heat-inactivated human AB serum supplemented with holotransferrin. IL-7 (1000 units/ml; Peprotech) was added at the initiation of cultures. On days 2 and 5 of culture, 20 IU/ml IL-2 (provided by Dr. Craig Reynolds, Biological Resources Branch, National Cancer Institute, Bethesda, MD) were added to each

[4] P. E. Gravitt, A. Hildesheim, R. Herrero, M. Schiffman, M. E. Sherman, M. C. Bratti, A. C. Rodriguez, L. A. Morera, F. Cardenas, F. P. Bowman, K. V. Shah, and P. A. Crowley-Nowick. Correlates of IL-10 and IL-12 concentrations in cervical secretions and association with HPV infection status, submitted for publication.

Table 1 Peptide pools used in cell cultures

E6 peptide pool 1	
1–15	M H Q K R T A M F Q D P Q E R
6–20	T A M F Q D P Q E R P R K L P
11–25	D P Q E R P R K L P Q L C T E
16–30	P R K L P Q L C T E L Q T T I
25–39	E L Q T T I H D I I L E C V Y
31–45	H D I I L E C V Y C K Q Q L L
38–52	V Y C K Q Q L L R R E V Y D F
44–57	L L R R E V Y D F A F R D L
59–72	I V Y R D G N P Y A V C D K
64–79	G N P Y A V C D K C L K F Y S K
71–85	D K C L K F Y S K I S E Y R H
E6 peptide pool 2	
76–90	F Y S K I S E Y R H Y C Y S L
81–95	S E Y R H Y C Y S L Y G T T L
90–103	L Y G T T L E Q Q Y N K P L
95–109	L E Q Q Y N K P L C D L L I R
98–112	Q Y N K P L C D L L I R C I N
111–125	I N C Q K P L C P E E K Q R H
116–130	P L C P E E K Q R H L D K K Q
121–136	E K Q R H L D K K Q R F H N I R
126–140	L D K K Q R F H N I R G R W T
131–145	R F H N I R G R W T G R C M S
137–152	G R W T G R C M S C C R S S R T
144–158	M S C C R S S R T R R E T Q L
E7 peptide pool 1	
1–15	M H G D T P T L H E Y M L D L
6–20	P T L H E Y M L D L Q P E T T
11–25	Y M L D L Q P E T T D L Y C Y
16–30	Q P E T T D L Y C Y E Q L N D
21–35	D L Y C Y E Q L N D S S E E E
26–40	E Q L N D S S E E E D E I D G
31–45	S S E E E D E I D G P A G Q A
36–50	D E I D G P A G Q A E P D R A
E7 peptide pool 2	
41–55	P A G Q A E P D R A H Y N I V
46–60	E P D R A H Y N I V T F C C K
51–65	H Y N I V T F C C K C D S T L
62–75	D S T L R L C V Q S T H V D
66–80	R L C V Q S T H V D I R T L E
71–85	S T H V D I R T L E D L L M G
76–90	I R T L E D L L M G T L G I V
86–98	T L G I V C P I C S Q K P

well. At day 7, cell-free supernatants were harvested and frozen at −20°C.

ELISA for IFN-γ. Supernatants from the cytokine induction assay were thawed and tested for IFN-γ using an IFN-γ ELISA kit (Endogen), following the manufacturer's instructions.

Selection of Specimens for Validation Study. For the present study, we selected 24 women from among the 873 participants in our AIS study for whom sufficient material was available after the above described assays were completed. From each of the 24 women selected, we obtained supernatants from each of the eight culture conditions used for the IL-2 bioassay and seven culture conditions used for the cytokine induction assay (15 specimens per women, total; tetanus was not included as a condition for the cytokine induction assay). For each of the 360 resulting specimens, blinded duplicate specimens were prepared, labeled, and shipped to Linco Research, Inc. (St. Charles, MO) for testing using the LINCOplex assay. The 720 specimens sent for testing were ordered in a strict fashion, so that half of the duplicates were included on the same plate as the original and the other half of the duplicates were located on different plates from the original specimen. This was done to permit an assessment of both within- and between-plate variability in the blinded duplicates.

In addition to the 720 specimens described above, we selected supernatants from two individuals used as laboratory controls in our studies, whose specimens had been stimulated with PHA (and, therefore, expected to have high levels of cytokines) on various occasions. We created two pools of supernatants from these two individuals, performed serial 2-fold dilutions of each of these two pooled specimens, in duplicate, and blindly included these known dilution specimens ($n = 30$) along with the 720 validation study specimens. This was done to permit an initial evaluation of whether results obtained were proportional to the known amounts of cytokine in the specimen. For logistical reasons (*i.e.,* to avoid exceeding a total of 750 specimen), 2-fold serial dilutions from 1:1 to 1:64 were performed for one of the pooled specimens whereas dilutions from 1:1 to 1:128 were performed for the second pooled specimen. The same media used in the cultures were used to dilute these specimens (R2E media containing 5% heat-inactivated human AB serum). As with the 720 specimens, two of the dilution series were included on the same plate, whereas the other two were randomly inserted on different plates.

LINCOplex Assay. At the time of this study, the LINCOplex assay was designed as a multiplex assay capable of simultaneously quantitating the following eight cytokines: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and TNF-α. Since that time, the assay was expanded to include three additional cytokines—IL-5, IL-12 (p70), and granulocyte macrophage colony-stimulating factor—but these three cytokines were not evaluated in the present study. The LINCOplex assay was conducted as per the manufacturer's instructions (Linco Research, Inc.). In brief, the assay is based on conventional sandwich assay technology. The antibody specific to each cytokine is covalently coupled to Luminex microspheres, with each antibody coupled to a different microsphere uniquely labeled with a fluorescent dye. The microspheres are incubated with standards, controls, and samples (25 μl) in a 96-well microtiter filter plate for 1 h at room temperature. After incubation, the plate is washed to remove excess reagents, and detection antibody, in the form of a mixture containing each of the eight antibodies, is added. After a 30-min incubation at room temperature, streptavidin-phycoerythrin is added for an additional 30 min. After a final wash step, the beads are resuspended in buffer and read on the Luminex^{100} instrument to determine the concentration of the cytokines of interest. All specimens received were tested in replicate wells (total number of test wells = 1500). Results were reported as the mean of the replicates.

Statistical Analysis. When evaluating the blinded serial dilution specimens included in our study, the ratio of the observed to expected cytokine level was estimated to evaluate the degree of deviation from linearity. The expected value for a given dilution was calculated by dividing by 2 the observed cytokine level for the next highest dilution.

When results from blind duplicate specimens for each cytokine were compared to evaluate intra-assay agreement, the nonparametric Spearman correlation coefficient was calculated using the continuous data (12). For each cytokine, the levels observed were categorized into quartiles based on the overall distribution of results for the conditions being evaluated. The quartile variables were used to estimate the exact agreement (%) and the kappa statistic (13). The kappa statistic evaluates the degree of agreement beyond chance alone. It ranges from 0 to 1, with 0 indicating no agreement beyond chance and 1

Table 2 Results obtained from blinded specimens included in 2-fold serial dilutions

Levels reported are the mean of duplicate specimens included blindly. Each of the duplicate specimens was tested in replicate. Specimens with levels below the detectable limit of the assay (3 pg/ml) were reported as 1.5 pg/ml (half of lowest detectable level).

Sample	Dilution	IL-1β		IL-2		IL-10		IFN-γ		TNF-α	
		Level (pg/ml)	O/E[a]	Level (pg/ml)	O/E	Level (pg/ml)	O/E	Level (pg/ml)	O/E	Level (pg/ml)	O/E
1	1:1	581		7270		232		9345		768.5	
1	1:2	292	1.01	6493.5	1.79	84	0.72	4412	0.94	380	0.99
1	1:4	137.5	0.94	2192	0.68	37.5	0.89	1805.5	0.82	172	0.91
1	1:8	74.5	1.08	894	0.82	14.5	0.77	942	1.04	77	0.90
1	1:16	37.5	1.01	339	0.76	3.75	0.52	490.5	1.04	29	0.75
1	1:32	19.5	1.04	124.25	0.73	1.5	NA	231	0.94	11	0.76
1	1:64	11	1.13	52.5	0.85	1.5	NA	109	0.94	5.5	1.00
2	1:1	1421.5		2397		322.5		1259.5		362	
2	1:2	697	0.98	986.25	0.82	121	0.75	704.5	1.12	148	0.82
2	1:4	372.5	1.07	504.5	1.02	54.5	0.90	386	1.10	71.5	0.97
2	1:8	210.5	1.13	205	0.81	22.5	0.83	213	1.10	35	0.98
2	1:16	109	1.04	65.5	0.64	6.5	0.58	100	0.94	14.5	0.83
2	1:32	65	1.19	29.5	0.90	1.5	NA	53.5	1.07	6	0.83
2	1:64	33	1.02	10.5	0.71	1.5	NA	29	1.08	1.5	NA
2	1:128	20.5	1.24	5	0.95	1.5	NA	14	0.97	1.5	NA

[a] O/E ratio, observed level divided by expected level, which is the level at the next highest dilution divided by 2; NA, not applicable because level is below the level of detection of the assay (*i.e.,* <3 pg/ml).

indicating complete agreement. Kappa values between 0.4 and 0.75 are typically considered good agreement, and values >0.75 are considered excellent agreement. Weighted kappa values are reported. Weights were assigned assuming that the category scores were on a linear scale. These weighted values account not only for whether disagreement is observed between duplicates, but also for the degree of disagreement. Thus, a duplicate pair where one result is categorized in the lowest quartile (Q1) and the second in the highest quartile (Q4) is treated more harshly than a duplicate pair where one result is classified in the lowest quartile (Q1) and the duplicate is categorized in the next highest quartile (Q2). Ninety-five percent CIs were calculated around our kappa estimates (13). In our analyses, specimens from the same individual tested under different conditions (*e.g.,* PHA, HPV16 E6, media alone, and others) were treated as independent variables. This is justified based on variance components analysis results demonstrating that the between condition variability was 4–40 times higher than the variability observed between individuals.

LINCOplex results for IL-2 and IFN-γ were also compared against previously available results for these same two cytokines using standard simplex assays. This allowed us to evaluate the agreement between assays for these two cytokines. The IL-2 comparisons were restricted to specimens obtained from the IL-2 bioassay cultures, whereas IFN-γ comparisons were restricted to specimens obtained from the cytokine induction cultures (see above). This was done because CTLL bioassay results for IL-2 and ELISA results for IFN-γ were only available on these subsets. As with the intra-assay analyses, these interassay analyses were conducted by estimating Spearman correlation coefficients, exact agreement (%), and weighted kappa values.

Analyses were conducted separately for all results (referred to as "overall results" and including the following conditions: PHA, FLU, tetanus, HPV antigens, and media only), results restricted to specimens that had been stimulated with HPV antigens (referred to as "results restricted to HPV conditions"), and results restricted to specimens that had been cultured without antigen stimulation (referred to as "results restricted to media condition"). These separate analyses were undertaken to evaluate the entire range of levels observed (overall), the range of levels one might expect in studies where immune responses to HPV or other exogenous antigens is of interest (results restricted to HPV conditions), and the range of values expected in unstimulated cells (results restricted to media condition). Responses to the mitogen PHA and the common recall antigens FLU (influenza) and tetanus are expected to be strongest and to generate the highest levels of cytokines, whereas response to HPV antigens is expected to be weaker but higher than that observed with the media alone. Assay performance often varies at different points in the range of detection of the assay, and so these separate analyses were felt to be of interest to reflect different ranges of results.

For two of the cytokines measured by the LINCOplex assay (IL-6 and IL-8), results were invariably high for our specimens. Results >10,000 pg/ml, the upper limit of detection of the assay, were observed for 65% and 99% of specimens for IL-6 and IL-8, respectively. For one of the cytokines measured by the LINCOplex assay (IL-4), results were invariably low for our specimens. Results ≤3 pg/ml, the lower limit of detection of the assay, were observed for 56% of specimens, and 90% of specimens had values <15 pg/ml. The lack of variability observed for these three cytokines precluded an analysis of assay performance. We have, therefore, chosen to focus our analyses on an evaluation of the remaining five cytokines measured by the LINCOplex assay (IL-1β, IL-2, IL-10, IFN-γ, and TNF-α).

Initial analyses examined specimens tested within and between plates separately. However, the results observed for these two group were nearly identical (Spearman correlations varied by no more than 0.07; range, 0.00–0.07), and so only results that combine these two groups are presented.

Results

Blinded Serial Dilutions. To independently validate the linearity of the assay within the reported range of detectability (3–10,000 pg/ml), we included two specimens at 2-fold serial dilutions ranging from 1:1–1:128. The two specimens selected were supernatants from cultures stimulated with PHA, a mitogen expected to induce strong lymphocyte responses and, there-

Table 3 LINCOplex cytokine levels: distribution and blind duplicate agreement

Percentage agreement and kappa statistics were computed after categorizing results for each cytokine into quartiles. Separate quartile distributions were computed for each of the analytical sets (*i.e.,* overall, HPV conditions only, and media conditions only).

Cytokine	No. of specimens	Median (pg/ml)	Range	Spearman correlation	Exact agreement	Weighted kappa [95% CI]
Overall results						
IL-1β	715	17	<3–6,719	0.96	87.6%	0.89 [0.86–0.92]
IL-2	707	38	<3–9,438	0.94	83.6%	0.85 [0.81–0.89]
IL-10	713	29	<3–762	0.95	77.6%	0.81 [0.78–0.85]
IFN-γ	712	175	<3–307,130	0.98	90.3%	0.92 [0.89–0.95]
TNF-α	704	95	<3–5,909	0.97	79.7%	0.83 [0.80–0.87]
Results restricted to HPV conditions						
IL-1β	382	14	<3–584	0.93	84.7%	0.85 [0.79–0.91]
IL-2	379	22	<3–465	0.92	77.5%	0.81 [0.75–0.87]
IL-10	380	22.5	<3–291	0.93	80.3%	0.84 [0.78–0.89]
IFN-γ	380	48.5	<3–2,569	0.98	84.6%	0.88 [0.84–0.92]
TNF-α	373	82	<3–1,436	0.97	79.6%	0.84 [0.79–0.89]
Results restricted to media condition						
IL-1β	94	11.5	<3–693	0.95	87.0%	0.88 [0.78–0.98]
IL-2	94	21.5	<3–342	0.86	78.3%	0.79 [0.67–0.92]
IL-10	96	17.5	<3–125	0.95	72.9%	0.79 [0.68–0.89]
IFN-γ	94	44.5	<3–1,225	0.97	78.3%	0.83 [0.73–0.93]
TNF-α	95	69	<3–534	0.97	80.9%	0.85 [0.75–0.94]

fore, high levels of cytokine production. Specimens were included and tested in duplicate; mean results are presented in Table 2. For the undiluted specimens, levels ranging from 232–9345 pg/ml were observed for the five cytokines evaluated. For both specimens and all five cytokines examined, the levels observed ranked in the appropriate order, with the undiluted specimen having the highest reported level and the specimen at the highest dilution (1:64 or 1:128) having the lowest level. When the ratio of observed to expected level was evaluated, most values were close to expectation (*i.e.,* close to 1.0) and did not vary from the expected by more than 15–20%. Some exceptions were noted, however, in particular for IL-2 and IL-10, where deviations from expectation of >20% were observed.

Intra-assay Reproducibility. We next evaluated the intra-assay reproducibility of the LINCOplex test for the five evaluable cytokines (Table 3). Overall, a wide range of levels was observed for these cytokines, permitting a careful evaluation of the assay for each of the five cytokines. The biggest range in reported values was seen for IFN-γ (median, 175 pg/ml; range, <3 pg/ml to 307,130 pg/ml). The smallest range in reported values was seen for IL-10 (mean, 29; range, <3 pg/ml to 762 pg/ml). For all five cytokines, Spearman correlation coefficients above 0.90 were observed (range, 0.94–0.98). Exact agreement ranged from 77.6–90.3%, when quartile distributions were examined. Kappa values for quartile distributions were in the excellent range, varying from 0.81 (95% CI, 0.78–0.85) for IL-10 to 0.92 (95% CI, 0.89–0.95) for IFN-γ.

As expected (see "Materials and Methods"), reported levels of cytokines were reduced when analysis was restricted to supernatants collected from cultures that contained HPV antigens, and were lowest when analysis was restricted to supernatants collected from cultures that had not been stimulated with antigen or mitogen. Despite these lower reported levels, the intra-assay reproducibility remained high, with Spearman correlation coefficients ranging from 0.92–0.98 for analysis restricted to HPV conditions and 0.86–0.97 for analysis restricted to media condition. Percentage exact agreement and kappa values ranged from 77.5–84.7% and 0.81–0.88, respectively, for analysis restricted to HPV conditions and from 72.9–87.0% and 0.86–0.97, respectively, for analysis restricted to media condition. For the HPV-restricted analyses, plots comparing the first and second value reported for each specimen are presented for each of the five cytokines in Fig. 1, *A–E*.

Comparison of LINCOplex and Standard Simplex Assay Results. Finally, for IL-2 and IFN-γ, we were able to compare results obtained using the LINCOplex assay against those previously obtained at an independent laboratory for those same specimens using standard simplex assays (IL-2 CTLL bioassay for measurement of IL-2, and Biosource ELISA for measurement of IFN-γ). Results of these comparisons are summarized in Table 4 and Figs. 2, *A* and *B.* Overall, very good interassay agreement was observed for both IL-2 and IFN-γ. Spearman correlations for IL-2 and IFN-γ of 0.86 and 0.93 were observed, respectively. Rates of exact agreement for IL-2 and IFN-γ were 68.5% and 67.3%, respectively, and kappa values were 0.72 and 0.73, respectively. Good interassay agreement was also observed in analyses restricted to HPV conditions (IL2: Spearman = 0.78, agreement = 56.7%, kappa = 0.58; IFN-γ: Spearman = 0.87, agreement = 59.8%, kappa = 0.65) and to media condition (IL2: Spearman = 0.78, agreement = 50.0%, kappa = 0.53; IFN-γ: Spearman = 0.93, agreement = 72.7%, kappa = 0.78).

Discussion

Results from the present study suggest excellent performance of the LINCOplex assay for the simultaneous measurement of multiple cytokines using culture supernatants from standard functional immunological assays. For the five evaluable cytokines (IL-1β, IL-2, IL-10, IFN-γ, and TNF-α), overall intra-assay correlation coefficients larger than 0.9 were consistently observed. Overall agreement rates and kappa coefficients were also high (exact agreement ranging from 77.6–90.3%, and kappas ranging from 0.81–0.92). For IL-2 and IFN-γ, when the results from the LINCOplex assay were compared against results obtained independently using standard simplex assays, good agreement between assays was also observed, suggesting that the assay is not only reproducible but also valid.

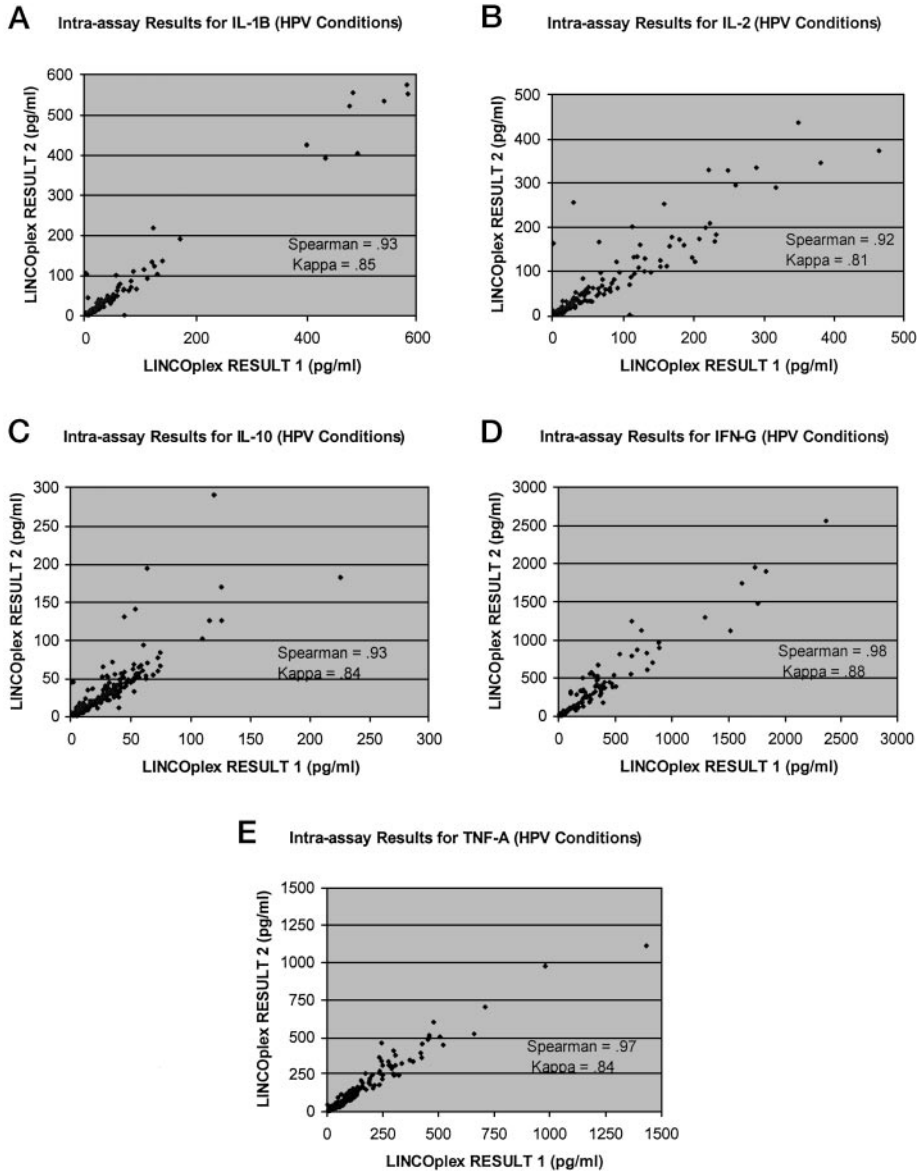

Fig. 1. Intra-assay results in the LINCOplex Study.

When analysis was restricted to results obtained using supernatants that had been derived from cultures stimulated with HPV antigens or no antigen at all, similar results were obtained to those seen in the overall analysis. This finding suggests the applicability of the LINCOplex assay under conditions where moderate or weak cytokine responses/levels are expected.

For two cytokines, IL-2 and IL-10, the nonlinearity of measured values observed for specimens included blindly at multiple known dilutions remains unexplained. This lack of linearity did not affect the intra- or interassay reproducibility of the test for these two cytokines, suggesting that the LINCOplex assay yields valid results when the primary objective is to compare readings from different specimens at a given concentration. This is the case, for example, in most epidemiological studies in which the absolute levels are not as important as relative values when comparing diseased and nondiseased groups. When knowledge of absolute levels for these two cytokines are of interest, however, caution should be used in interpreting results from these assays.

In analysis restricted to HPV conditions, when the LINCOplex assay was compared against the standard IL-2 CTLL bioassay, good agreement was observed overall, but we did note very discrepant results for 7 of the 90 specimens evaluated (Fig. 2*A*). On careful evaluation of these individual specimens, we noted that all of the seven discrepant results occurred for specimens obtained from cultures that included the same pool of HPV antigens. Because the results obtained by the standard IL-2 CTLL bioassay for these seven specimens was 7–10 times higher than the median levels observed for the other specimens tested using this assay, we suspect that the discrepancies observed were due to problems in our IL-2 CTLL bioassay for those specific specimens rather than problems with the LINCOplex assay.

Although IL-4, IL-6, and IL-8 testing was performed in our study, we were not able to evaluate the LINCOplex assay

Table 4 Comparison of results from LINCOplex and standard simplex assays

	IL-2		IFN-γ	
	LINCOplex (pg/ml)	IL-2 CTLL (cpm)	LINCOplex (pg/ml)	ELISA (pg/ml)
Overall (n = 178 for IL-2 and 162 for IFN-γ)				
Median	121	27,105	392.3	259.8
Range	7–8,547	68–392,987	16–278,210	<15.6–>1,000
Spearman correlation	0.86		0.93	
Exact agreement	68.5%		67.3%	
Weighted kappa [95% CI]	0.72 [0.65–0.79]		0.73 [0.67–0.80]	
Results restricted to HPV conditions (n = 90 for IL-2 and 92 for IFN-γ)				
Median	68	15,667	275.5	185.0
Range	7.5–419.5	68–186,679	16–2,468	<15.6–>1,000
Spearman correlation	0.78		0.87	
Exact agreement	56.7%		59.8%	
Weighted kappa [95% CI]	0.58 [0.46–0.70]		0.65 [0.55–0.75]	
Results restricted to media condition (n = 22 for IL-2 and IFN-γ)				
Median	49	10,535	241.3	162.5
Range	7.5–189	156–62,607	16–1,172	<15.6–>1,000
Spearman correlation	0.78		0.93	
Exact agreement	50.0%		72.7%	
Weighted kappa [95% CI]	0.53 [0.28–0.77]		0.78 [0.61–0.94]	

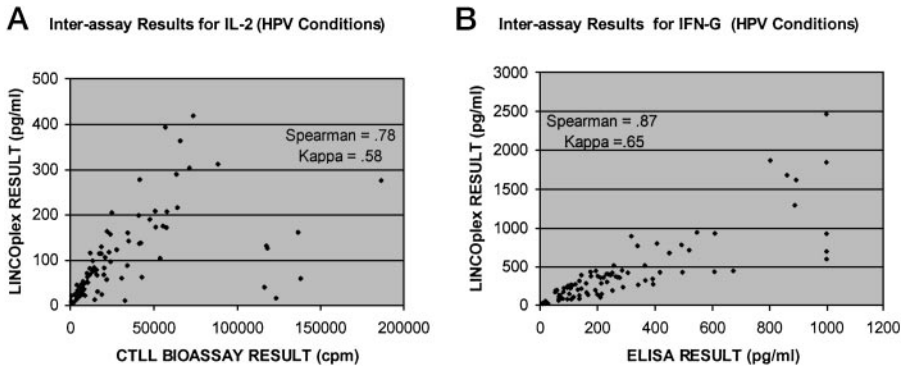

Fig. 2. Inter-assay results in the LINCOplex Study.

for the measurement of these three cytokines because results were either invariably low (IL-4) or invariably high (IL-6 and IL-8). These observations highlight an important point for those interested in using multiplex assays for the measurement of cytokines in the future. Because the range in levels of these various cytokines varies, the ideal dilution to be used for testing might vary from cytokine to cytokine. For example, for both IL-6 and IL-8, testing at a greater dilution would likely have brought the results into the linear range of the assay, permitting a more careful evaluation of the assay for these two cytokines. However, having to run the multiplex assay multiple times at different dilutions could eliminate one of the advantages of using a multiplex assay in the first place. To partially address this problem, we have chosen the following strategy for our future work. First, we will select a random subset of specimen from the study of interest and will have them tested (undiluted) for the cytokines of interest using the multiplex assay. This will allow us to determine the range in observed levels for each cytokine within the study of interest. On the basis of these initial findings, the cytokines of interest will be divided into two (or at most three) groups, one requiring testing at a low dilution and the other testing at a higher dilution. Parallel testing for these two sets of cytokines using specimens at different dilutions would then be possible while still permitting the evaluation of a larger number of cytokines than would have been possible using standard simplex assays. For the LINCOplex assay, this approach is feasible because the assay kits were specifically developed to allow for tailoring of the kit for specific subsets of cytokines of interest.

The present study has demonstrated the usefulness of the LINCOplex assay for the measurement of cytokines using small amounts of culture supernatants obtained from standard functional immunological assays. Given that this specimen matrix is similar to that of serum, one might predict that the assay would also perform well in studies where direct testing of serum is of interest. It remains to be determined, however, whether the LINCOplex assay is also suitable for testing more complex specimen matrices such as mucosal specimens (*e.g.*, cervical secretions or saliva). Historically, mucosal specimens have proven more difficult to work with than blood or culture supernatants. Work is ongoing in our group to evaluate this question.

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